SAHA-induced TRAIL-sensitisation of Multiple Myeloma cells is enhanced in 3D cell culture

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SAHA-induced TRAIL-sensitisation of Multiple Myeloma cells is enhanced in 3D cell culture.

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Abstract

Background: Multiple Myeloma (MM) is currently incurable despite many novel therapies. Tumour Necrosis Factor-Related Apoptosis-Inducing Ligand (TRAIL) is a potential anti-tumour agent although effects as a single agent are limited. In this study, we investigated whether the Histone Deacetylase (HDAC) inhibitor SAHA can enhance TRAIL-induced apoptosis and target TRAIL resistance in both suspension culture, and 3D cell culture as a model of solid disseminated MM lesions that form in bone.

Methods: The effects of SAHA and/or TRAIL in 6 Multiple Myeloma cell lines were assessed in both suspension cultures and in an Alginate-based 3D cell culture model. The effect of SAHA and/or TRAIL was assessed on apoptosis by assessment of nuclear morphology using Hoechst 33342/Propidium Iodide staining. Viable cell number was assessed by CellTiter-Glo luminescence assay, Caspase-8 and -9 activities were measured by Caspase-Glo^{TM} assay kit. TRAIL-resistant cells were generated by culture of RPMI 8226 and NCI-H929 by acute exposure to TRAIL followed by selection of TRAIL-resistant cells.

Results: TRAIL significantly induced apoptosis in a dose-dependent manner in OPM-2, RPMI 8226, NCI-H929, U266, JJN-3 MM cell lines and ADC-1 plasma cell leukaemia cells. SAHA amplified TRAIL responses in all lines except OPM-2, and enhanced TRAIL responses were both via Caspase-8 and -9. SAHA treatment induced growth inhibition that further increased in the combination treatment with TRAIL in MM cells. The co-treatment of TRAIL and SAHA reduced viable cell numbers all cell lines. TRAIL responses were further potentiated by SAHA in 3D cell culture in NCI-H929, RPMI 8226 and U266 at lower TRAIL + SAHA doses than in suspension culture. However TRAIL responses in cells that had been selected for TRAIL resistance were not further enhanced by SAHA treatment.

Conclusions: SAHA is a potent sensitizer of TRAIL responses in both TRAIL sensitive and resistant cell lines, in both suspension and 3D culture, however SAHA did not sensitise TRAIL-sensitive cell populations that had been selected for TRAIL-resistance from initially TRAIL-sensitive populations. SAHA may increase TRAIL sensitivity in insensitive cells, but not in cells that have specifically been selected for acquired TRAIL-resistance.

Keywords: Apoptosis, cell proliferation, Multiple Myeloma, SAHA, TRAIL.

1. Introduction

Multiple Myeloma (MM) is considered the most common blood malignancy in the USA after non-Hodgkin lymphoma [1] accounting for 1% of the malignant diseases [2] and approximately 10% of all blood cancers. It is characterized by a proliferation of malignant B cells in the bone marrow [3]. The common clinical manifestations of disease are lytic bone disease, renal impairment, haematological abnormalities, peripheral neuropathy and pathological fractures and recurrent infection [3].

Despite the considerable improvements of the available chemotherapeutic agents, MM remains an incurable disease with a high mortality rate due to the persistence of drug-resistant tumour cells. Therefore, an urgent need arises to find alternative therapeutic strategies that eradicate all tumour cells [4].

Binding of TRAIL to Death Receptor-4 or -5 (DR4 and -5) triggers apoptosis by activation of caspase-8, resulting in activation of executioner caspase-3, -6 or -7 (extrinsic pathway) [5] Caspase-8 may also cleave BID to tBid to activate the intrinsic pathway, facilitating release of pro-apoptotic cytochrome c from the mitochondria and activating pro-Caspase-9, thus linking the extrinsic to the intrinsic apoptotic signalling pathways [6].

Clinical trials to assess the safety and anti-cancer activity of Tumour Necrosis Factor-Related Apoptosis-Inducing Ligand (TRAIL) and agonistic Death Receptor antibodies have been completed in several tumour types [7-9] including MM [10]. Unfortunately, TRAIL resistance develops in vitro [11] and in clinical trials, TRAIL insensitivity may be present in most tumours [12]. Consequently combined therapy of TRAIL with other anti-tumour agents is required to restore the TRAIL sensitivity [9].

Growing evidence suggests that epigenetic alterations play an essential role in the down-regulation of tumour suppressor genes and up-regulation of oncogenes in the initiation and progression of many types of cancers as reported by a number of studies [13]. The chromatin acetylation is controlled by histone acetyltransferases (HAT) and histone deacetylases (HDAC) [14]. These regulate the histone acetylation status, resulting in altering the chromatin structure and altered gene expression. HDAC inhibitors ($HDAC^{i}$) inhibit HDACs, resulting in histone hyper-acetylation status that facilitate the DNA accessibility and binding to transcriptionally co-activating factors and consequent in regulating gene expression involved in regulating growth, differentiation, and apoptosis [15].

HDAC^{i} have been recently proposed as potential therapeutics for MM [16,17]. However it is well known that HDAC^{i} are potential TRAIL-sensitizers with the ability to overcome TRAIL-resistance in other tumour types [18]. Previous studies on SAHA have previously been shown to enhance TRAIL-responses in Lymphoma [19] and T-cell lymphoblastic leukaemia [20]. A single study has previously demonstrated enhanced TRAIL-induced apoptosis by combination treatment with SAHA in MM 2 cell lines, showing up-regulation of pro-apoptotic proteins, including Bak, Bim, Bax, PUMA and Noxa and down-regulation anti-apoptotic proteins such as Bcl-2 and Bcl-xL [21].

The development of experimental tools to facilitate the study of myeloma cell biology and susceptibility to therapeutic agent is considerably limited due to the lack of *in vitro* model systems that permits myeloma cells reproducible growth as well as the putative cancer stem cell compartment. The development of a 3-dimensional (3D) model systems have recently been proposed to support *in vitro* myeloma cells expansion, facilitating modelling of *in vivo* responses [22]. In this study, we investigated the effect of the HDAC^{i} SAHA with TRAIL on 5 established human multiple myeloma cell lines and one primary plasma cell leukaemia cell culture in both suspension conditions and 3D cell culture, using the Alginate bead assay.

2. Materials and methods

2.1. MM Cell Lines

Five Human Multiple Myeloma cell lines (NCI-H929, RPMI 8226, OPM-2, JJN-3, and U266) and one primary cell culture generated from a case of plasma cell leukaemia (ADC-1). **NCI-H929** was originally obtained from the pleural fluid of a 67 year old Caucasian female with an IgA-producing plasmacytoma (European Collection of Cell Cultures (ECACC), Salisbury, UK; cat no 95050415). **RPMI 8226** was originally obtained from the peripheral blood of a 61year old man with multiple myeloma at diagnosis (ECACC, Salisbury, UK; cat no 87012702). **OPM-2** was originally obtained from the peripheral blood of a 56 year old woman with multiple myeloma in terminal leukaemic phase (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Braunschweig, Germany; DSMZ no ACC 50). **JJN-3** was originally obtained from a plasma cell leukaemia (kind gift from Professor I. Franklin, University of Glasgow, UK). **U266** was originally derived from the peripheral blood of a 53-year-old man with MM) and purchased from LGC Standards (UK). **ADC-1** was obtained from the peripheral blood of a patient with plasma cell leukaemia presenting to the Dept. of Haematology, Sheffield Teaching Hospitals. Patient cells were acquired with appropriate ethical permission (REC reference: 05/Q2305/96). This consent procedure was approved by the South Sheffield Research Ethics Committee.

2.2. Culture Conditions

MM cell lines were cultured in the RPMI-1640 medium + L-Glutamine supplemented with 10% foetal calf serum, 1% penicillin–streptomycin and 1% non-essential amino acid and incubated under standard cell culture conditions at 37ºC with 5% CO_2 atmosphere.

2.3. CellTiter-Glo® Luminescent Cell Viability Assay

The CellTiter-Glo® Luminescent Cell Viability Assay Kit (Promega, Southampton, UK) was used to assess the relative number of live cells based on the quantification of ATP levels. MM Cells were plated at 350,000 cells/ml in a 96-well plate and treated with SAHA (Sigma, Poole, UK) at 0-10 µM in the presence or absence of 0-50ng/ml TRAIL (PeproTech EC Ltd (London, UK) for 24h. All treatments were performed in triplicate, in three independent experiments. Following treatments, cellular proliferation was measured as per manufacturer's instructions using Wallac Victor 2 1420 luminometer.

2.4. Assessment of the apoptosis

2.4.1. Hoechst 33342 and Propidium Iodide (PI) *Nuclear Morphological Analysis by Fluorescence Microscopy*

Following treatment with SAHA and/or TRAIL, induction of apoptosis was assessed using Hoechst 33342 and Propidium Iodide (PI) staining (Sigma-Aldrich, Dorset, England). Cell were stained with 10µg/ml Hoechst 33342 and 10µg/ml PI for 30 min at 37ºC and examined using an IX81 fluorescence microscope (Olympus) and images captured using Cell-F software (Olympus). In some experiments, 10µM of the caspase-3 inhibitor Z-DEVD-FMK was co-incubated with TRAIL treatments. Apoptotic cells were counted manually and percent apoptosis calculated based on duplicate representative fields of view each containing at least 100 cells for three independent experiments.

2.4.2 NucView Caspase 3 Activity Assay by Flow Cytometry

To confirm apoptotic responses of SAHA and TRAIL at doses that exhibited synergistic induction of apoptosis with Hoechst 33342/Propidium Iodide staining, NucView Caspase-3 activity assay (Biotium, Cambridge Biosciences, Cambridge, UK) was used. As a negative control, 10µM of caspase-3 inhibitor Z-DEVD-FMK was added (R&D systems). Following treatment, 200µl of each cell suspension was transferred to a flow cytometry tube and 2.5µL of Nucview Caspase-3 substrate (0.2mM) was added to the sample (including inhibitor sample) except 'unstained control' and incubated cells at room temperature for 20 minutes. Finally, the samples were analysed on the flow cytometer using a Beckman Coulter Gallios flow cytometer. Ten thousand events were acquired per sample.

2.4.3 Assessment of Caspase-8 and -9 activities

Caspase-8 and -9 activities in MM cells were determined using the Caspase-Glo® 8/9 Assay (Promega, Southampton, UK) according to manufacturer's instructions. Cells were lysed as Caspase-Glo reagent added, followed by detection of luminescent signals due to caspase cleavage of the substrate. Briefly, MM cell lines were treated with SAHA, TRAIL or both, in white 96 well-plates (Fisher Scientific). Following 24 hours incubation, 50µl of Caspase-Glo 8 Reagent or Caspase-Glo 9 Reagent (Promega) with the proteasome Inhibitor MG-132 to reduce nonspecific background activity were added to each well. After incubation for 90 minutes at RT luminescence was measured using Wallac Victor 2 1420 luminometer.

2.5 Induction of 3D tumour spheroid formation using Alginate bead culture

To establish 3D alginate cultures, MM at a density of $1x10^6$ cells/ml were re-suspended in a final concentration of 1.2% (w/v) medium-viscosity sodium alginate dissolved in 0.15M saline (Sigma, Poole, UK). The alginate-cell suspension was extruded through a 19-gauge needle into 200mM $CaCl_2$ and incubated at 37°C for 15 minutes to polymerise the alginate spheres. Following incubation, beads were washed twice in 0.15M NaCl, then washed in growth medium [23]. Alginate beads were cultured in growth medium as per suspension cells and medium changed every 3-4 days. After 10 days culture in alginate beads, MM cells were treated with SAHA or 0.1% DMSO (vehicle control) in the presence or absence of TRAIL for 24 hours, as described earlier. Colonies of MM cells were released from the alginate by incubation with 150mM NaCl, 55mM sodium citrate and 20mM EDTA, which chelates the Ca2+ ions. Apoptotic morphology of cells within released colonies was assessed by staining with Hoechst 33342/PI staining (10µg/ml of each for 30mins). All treatments were performed in triplicate and in each experiment, assessment of apoptosis in at least 10 individual colonies assessed. In some experiments, 10µM of the caspase-3 inhibitor Z-DEVD-FMK was co-incubated with combination treatments. Images were taken on an Olympus IX81 inverted microscope running Cell-F software.

2.6. Generation of TRAIL-resistant Multiple Myeloma cells in vitro

In order to generate TRAIL-insensitive cells NCIH-929, RPMI 8226, and OPM2 were seeded into T25cm^2 flasks (Invitrogen, Paisley, UK) and grown in in escalating doses of TRAIL for 1 year. Cell viability in response to TRAIL was checked every week and the dose of TRAIL is increased depending on cell viability. TRAIL-resistant cells were generated by culture of RPMI 8226 and NCI-H929 in escalating doses of TRAIL for 1 year, and also by acute exposure of the TRAIL sensitive cells with a high/lethal dose of TRAIL followed by selection of TRAIL-resistant cells. The cytotoxic activity of TRAIL was determined on NCIH-929, RPMI 8226, and OPM2 and compared to cell isolated from parental TRAIL-sensitive NCIH-929 and RPMI 8226 culture. Live cells counts were determined using a Cell Countless system (Invitrogen, Paisley, UK) and cell viability was determined using trypan blue staining (Invitrogen, Paisley, UK). Growth inhibition response to anti-tumour agents was assessed and apoptosis was assessed using CellTiter-Glo® luminescent assay and Hoechst 33342 staining of nuclear morphology.

2.9. Statistical Analysis

Data are expressed as the mean ± SD. Shapiro Wilke test using Stats Direct software (Stats Direct Ltd, England) was used for analysis whether data followed a normal distribution. Data which did not follow a normal distribution, Kruskal–Wallis one-way analysis of variance and Connover-Inman post hoc was using to investigate significant differences. $P < 0.05$ was considered statistically significant. The SAHA-mediated potentiation of TRAIL-induced apoptosis was determined by showing that apoptosis was induced by a combined treatment which was significantly greater than additive (i.e., apoptosis resulting from co-treatment with TRAIL and SAHA was significantly greater than the sum of apoptosis induced by TRAIL alone along with apoptosis induced by SAHA alone, after removal of background apoptosis from all values).

3. Results

3.1. Assessment of apoptosis using Hoechst 33342 and PI nuclear staining

3.1.1 Effect of TRAIL Treatments on MM cell lines

MM cell lines (NCI-H929, RPMI 8226, OPM-2, JJN-3, U266 and ADC-cells) were treated with TRAIL (0–50 ng/ml) for 24h. OPM-2 MM cells were most sensitive to TRAIL (50 ng/ml) followed by RPMI 8226, NCI-H929, and ADC-1 cell lines which also showed significant apoptotic responses. In contrast U266 and JJN-3 were less sensitive to TRAIL but showed significant induction of apoptosis although observed apoptosis was less than 20% at 50 ng/ml $(P \leq 0.05)$ (Figure 1). NCI-H929, RPMI 8226, U266 and JJN-3 showed dose-dependent increases in apoptosis up to 250ng/ml TRAIL, which was almost completely inhibited by the caspase-3 inhibitor Z-DEVD-FMK (Supplementary Fig 1).

3.2. The effect of SAHA in combination with TRAIL on apoptosis in MM cell lines

MM cells were treated with TRAIL at the lowest dose that induced significant apoptosis as a single treatment, combined with SAHA. Hoechst 33342 and PI staining of nuclear morphology confirm that SAHA enhances the apoptotic activities of TRAIL in MM cell lines (Figure 2a). SAHA significantly induced apoptosis of MM cells in a dose-dependent manner in all MM cell lines at though effect sizes were smallest in JJN-3 and OPM-2 (Fig 2b). SAHA combined with TRAIL synergistically induced apoptosis in RPMI 8226 (10μM SAHA), NCI-H929 (5 and 10μM SAHA), U266 (5 and 10μM SAHA), JJN-3 (5 and 10μM SAHA) and ADC-1 (10μM SAHA),when using sub-toxic doses of TRAIL in each cell line (Figure 2b), however the size of these effects was modest except for JJN-3 in suspension culture.

3.3. Effects of SAHA in combination with TRAIL on the viability in MM cell lines

The cytotoxic effects of SAHA on myeloma cell lines NCI H929, RPMI 8226, OPM-2, JJN-3, U266 and ADC-1 cells following treating with increasing concentrations of SAHA for 24 hours were studied using CellTiter-Glo® Luminescent Cell Viability Assay. Viability assays identified that treatment with SAHA for 24h resulted in reduced viable cells in MM cell lines in a dose dependent manner (Fig. 3) consistent with observed induction of apoptosis as shown in figure 2. SAHA alone significantly reduced cell numbers in all cell lines. TRAIL was used at the lowest dose that resulted in significant apoptosis by Hoechst 33342/PI

staining, and as such, weak effects seen with TRAIL alone are expected. Combination treatment with SAHA + TRAIL resulted in a significant reduction in viable cells, consistent with observations of apoptotic morphology shown in figure 2.

3.4. Effect of HDAC^{i} SAHA on Caspase activity in MM cells

To confirm morphological assessment of apoptosis by Hoechst 33342/PI staining, caspase-3 activity assays using flow cytometry was used. Combination treatment doses which synergistically enhanced apoptosis as determined by Hoechst 33342/PI staining showed significant induction of apoptosis as shown by increased caspase-3 activation (Figure 4a) in NCIH 929, U266 and RPMI 8226**.** Moreover, to determine which apoptotic pathway was responsible for SAHA-mediated enhanced TRAIL-signalling, the activity of Caspase-8 (early initiator caspase of the extrinsic pathway), caspase-9 (early initiator caspase of the intrinsic pathway) was measured with cell-based homogenous Caspase-Glo kit assay after exposure of NCIH 929, U266 and RPMI cells to SAHA for 24 hours and compared with vehicle control. As expected, TRAIL alone (2ng/ml) enhanced caspase-8 and caspase-9 activity. SAHA also enhanced both Caspase-8 and -9 activities, and combination treatment further elevated caspase-8 and -9 consistent with morphological observations. The results confirm that both TRAIL and SAHA significantly increased both cellular caspase 8/9 activities and that enhanced apoptosis from dual treatment is in part via the intrinsic pathway.

3.5 SAHA does not enhance TRAIL-sensitivity in TRAIL-resistant cell lines

In order to facilitate the development of TRAIL-resistant culture, we intentionally chose growth conditions which support the appearance of resistant clones by long-term culture in TRAIL following exposure of cells to acutely toxic doses of TRAIL and sub-culture of surviving cells. Measurement of cell number and viability confirmed the TRAIL-insensitive phenotype in NCI-H-929, and RPMI 8226 with highly significant increase of cell viability of TRAIL-resistant cells compared to parental TRAIL sensitive cells in response to high-dose TRAIL (Fig 5a-b, $p<0.0001$). Treatment of TRAIL-resistant NCI-H929 with 250ng/ml TRAIL induced <10% apoptosis vs. >90% apoptosis in unselected cells (Supplementary figure 2). Moreover, treatment of the TRAIL-resistant cells with 10 µM SAHA either alone or in combination with TRAIL result in significant reduction of apoptosis in TRAIL resistant cells compared to parental TRAIL sensitive cells or vehicle control (Figure 5 (b)).

3.6. SAHA-induced TRAIL-sensitisation is enhanced in 3D cell culture

The effect of SAHA on TRAIL-induced apoptosis in 3D cell culture vs. suspension culture was studied using Hoechst 33342 staining of nuclear morphology. MM cells line were treated with 1 μM SAHA or 0.1% DMSO vehicle control either alone or in combination with TRAIL (50ng/ml for U266 and 2ng/ml for NCI-H929) for 24 hours. We observed that MM were more sensitive to SAHA alone in 3D culture conditions compared to suspension cultures and there was significant increase in TRAIL-induced apoptosis by SAHA treatment in U266 and NCIH-929 cell lines ($P \leq 0.0001$) (Figure 6). However, SAHA (1 μM) has no significant effect on apoptosis of U266 and NCIH929 cells in suspension cultures. Co-treatment of TRAIL + SAHA-treated 3D cultures with the caspase-3 inhibitor Z-DEVD-FMK completely inhibited apoptosis to control levels (Supplementary figure 3).

4. Discussion

In spite of the successful introduction of a number of novel agents, MM still remains a predominantly incurable disease. Relapse occurs due to the eventual emergence of plasma cell clones resistant to the currently used chemotherapeutic agents. Therefore, an urgent need arises to find therapies that eradicate all tumour cells [24]. Recently, the therapeutic potential of TRAIL-based therapy both *in vivo* and *in vitro* against various tumour cells including MM cells suggests that it may be a promising anti-myeloma therapeutic candidate. However, the susceptibility of MM cells to TRAIL-based therapy has been established to be low in most of the MM cells line, which limits TRAIL clinical applications [25]. This study aimed to investigate the susceptibility and resistance of the MM cells line to apoptosis mediated by TRAIL-based therapies in addition to the combined treatment of TRAIL with other chemotherapeutic therapeutic agents in order to overcome the TRAIL resistance. As a result,

we have demonstrated that TRAIL induces apoptosis in some myeloma cells lines. Importantly we have shown that TRAIL treatment in combination with SAHA results in sensitising some Multiple Myeloma cell lines to TRAIL. Moreover, SAHA induces more apoptosis of MM cells with more potent synergistic effect in 3D culture conditions, even in cells that do not respond to either agent in suspension culture.

SAHA enhances TRAIL-Induced Apoptosis and Cytotoxicity in Multiple Myeloma cell lines

HDAC inhibitors are a class of anti-tumour agents that are able to induce apoptosis and/or cell cycle arrest in tumour cells although the under lying anti-tumour effects of molecular mechanisms are not fully understood. Due to its *low* toxicity SAHA activity is currently evaluated in clinical trials [21]. In this study, we have evaluated the effect of the HDAC inhibitor SAHA on human multiple myeloma cell lines. The findings established that together with the SAHA induced apoptosis and growth arrest, the enhanced apoptotic and the cytotoxic effects of TRAIL on U266 and NCI H929 (10 ng/ml TRAIL+ 5 or 10 μM SAHA) cell lines (Figures 2, 3). Additionally, the co-treatment with SAHA with TRAIL enhanced cytotoxicity as determined by CellTitre-Glo assay (Figure3).

Despite considerable interest in HDAC^{i} for MM therapy, few studies have addressed their role in combination with TRAIL-based therapies, and only one previous study has addressed any HDAC^{i} with TRAIL in MM cells [18]. The results here are consistent with a study carried out previously whereby two HDAC inhibitors SAHA and TSA induced growth arrest and apoptosis in human multiple myeloma cell lines and that the augmented apoptotic and cytotoxic effects of TRAIL, albeit not in this cell panel [18]. SAHA and TSA induced the transcription and the expression of surface TRAIL DR4/DR5 death receptors, up-regulated the expression of pro-apoptotic protein and down-regulated the expression of other anti-apoptotic proteins Bcl-2 and IAPs. Moreover, the cytotoxic effect of SAHA on MM cell lines may be in part caspase-independent and was mediated via the release of mitochondrial AIF [21].

Consistent with these findings, Rosato *et al* (2015) [26] demonstrated that that co-administration of TRAIL with HDAC inhibitors SAHA or the HDAC^{i} sodium butyrate synergistically induces apoptosis in human myeloid leukemia cells and provide further evidence that TRAIL/HDAC Inhibitor activate both the extrinsic and intrinsic pathways of

apoptosis by activation of Caspase-3 and -8. Moreover, TRAIL/HDAC^{i} mediated apoptosis in leukemic cells is associated with cleavage of Bid and Bcl-2, down-regulation of XIAP, moderate reduction on Bcl-XL, and cytosolic depletion of pro apoptotic Bax as well as cytoplasmic release of cytochrome c, AIF, and Smac/DIABLO [26]. The mechanisms by which SAHA enhances apoptosis in these cells are still largely unclear. This study examined the relation SAHA and the activities of cellular caspase-3, -8 and -9 in three MM cell l lines. It was found that HDAC inhibitors SAHA can activate caspases-3, -8 and -9 in these cell lines independently of TRAIL stimulation (Figure 4). Although it is acknowledged that caspase-3 in particular may cleave other caspases [27], caspase-8 and -9 were induced at doses of SAHA where caspase-3 positivity was low NCI-H929, and differential responses were seen in, in that caspase-9 was induced in NCI-H929, but not U266.

Prolonged incubation of TRAIL sensitive MM cells with TRAIL reduced their sensitivity to SAHA

The anti-tumour potential of TRAIL either alone or in combination has been established in various *in vivo* models of tumour growth. However, the reported treatment efficacies are variable from tumour to tumour. This may reflect many factors however the presence of pre-existing TRAIL-resistant cells and the emergence of TRAIL-resistance during therapy both contribute to treatment failure. To address the latter cause of TRAIL-insensitivity, we specifically aimed to develop TRAIL-resistant cell lines with the aim of determining whether TRAIL-sensitizers, such as SAHA are able to enhance TRAIL sensitivity in these cells, since it is known that SAHA can enhance TRAIL sensitivity on cells with pre-existing TRAIL insensitivity.

In the current study, we incubated the TRAIL sensitive cells (NCI-H929, RPMI 8226) with an acutely toxic dose of TRAIL and sub-cultured surviving cells long term. Surviving cell populations predictably demonstrated increased TRAIL-insensitivity (Figure 5 a-b). NCI-H929 showed decreased SAHA sensitivity after selection for TRAIL-resistance, and both cell lines demonstrated a loss of SAHA-mediated sensitisation to TRAIL (Figure 5 cd). Consistent with these observations, Vitovski et al. (2012) [24], demonstrated that the prolonged incubation with TRAIL in MM cells results in the emergence of TRAIL-resistant CD138-negative cells and reduce the cell toxicity in TRAIL resistant cells compared to the parental, TRAIL-sensitive culture. TRAIL-resistant cells were not necessarily more

apoptosis-resistant *per se*, and indeed, TRAIL-resistant cells showed increased sensitivity to selected small molecule inhibitors including histone methyltransferase suggesting that these cells showed attenuated responses for death receptor-mediated apoptosis (data not shown). Analysis of gene expression profiling of TRAIL resistant cells did show significantly reduced DR4 and DR5 transcriptional expression in NCI-H929 TRAIL-resistant cell lines (9- and 5-fold respectively), however counter-intuitively, this was not observed in RPMI 8226, where DcR1 was significantly increased (10-fold) (data not shown).

3D cell culture enhances responses to both TRAIL and SAHA

Micro-environmental conditions, such as hypoxia within solid tumours are known to regulate tumorigenesis. Biomimetic culture systems may allow for *in vitro* tumour modelling to exploit cancer cells' dependency on these conditions [28]. Although MM is a haematological malignancy, it forms solid lesions in bone which are chronically hypoxic. Here we used 3D tumor models based on alginate gel to in-part mimic the hypoxic nature of solid MM lesions *in vitro*, although hypoxic chambers may mimic part of the tumour microenvironment. Our result show significantly higher sensitivity of MM cells grown as 3D spheroids in alginate gel scaffolds compared to suspension cell culture. TRAIL-sensitive RPMI 8226 and TRAIL-insensitive U266 were challenged with SAHA+/- TRAIL with more potent synergistic effects observed compared to suspension cultures (Fig 6). These results are consistent with a study carried out by Amatangelo *et al*., [29] that showed that the EZH2 methyltransferase inhibitors GSK343 significantly inhibited the growth and induces apoptosis of Epithelial ovarian cancer (EOC) cells cultured in 3D Matrigel extracellular matrix (ECM), which more closely mimics the tumour microenvironment in vivo but not standard 2D culture model. These results suggest that agents which display little effectiveness in conventional 2D or suspension culture models may still have therapeutic value within the solid ECM tumour microenvironment.

Other studies reported that spheroids cancer stem cell (CSC) in 3D alginate beads display increased resistance to anti-cancer agents due to enhance the tumour metastatic potency by increasing the expression of a number of matrix metalloproteinases (MMPs) including MMP2 and MT1-MMP in 3D-algenate cultured cells as well as the improvement of hypoxia through enhance the expression of hypoxia-inducible factors (HIFs) [30]. Together, these findings suggest that targeting the cell spheroids in 3D culture system is a novel approach to assessing the anti-tumour therapeutics, which are urgently needed.

In conclusion, we have shown that MM cells were more sensitive to anti-tumour agents in 3D alginate model with a synergistic effect with TRAIL. Importantly, our findings suggest that these anti-tumour agents may potentially enhance the action of TRAIL and that the combined treatment could possibly result in reducing the treatment doses, avoiding toxicity and using a novel anti-cancer agent for multiple myeloma. Toward this goal, future studies will investigate in more detail how the 3D alginate beads enhance the anti-tumour effect of HDAC-inhibitor SAHA in MM cells cultured in 3D vs. suspension conditions.

CONFLICT OF INTEREST

The author(s) confirm that the article content has no conflict of interest.

ACKNOWLEDGEMENTS

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Figures

Figure 1*: Six MM cell lines were treated with 0-50 ng/ml TRAIL for 24 h. The treatment with TRAIL potently induces apoptosis of MM cells in a dose-dependent manner. U266 and JJN3 were less sensitive to TRAIL treatment compared to other MM cell lines. Data are expressed as the median and standard deviation of 3 independent experiments. Statistical significance was determined by comparison treatment with the control, statistical significance was set at p<0.05=* and determined by ANOVA*

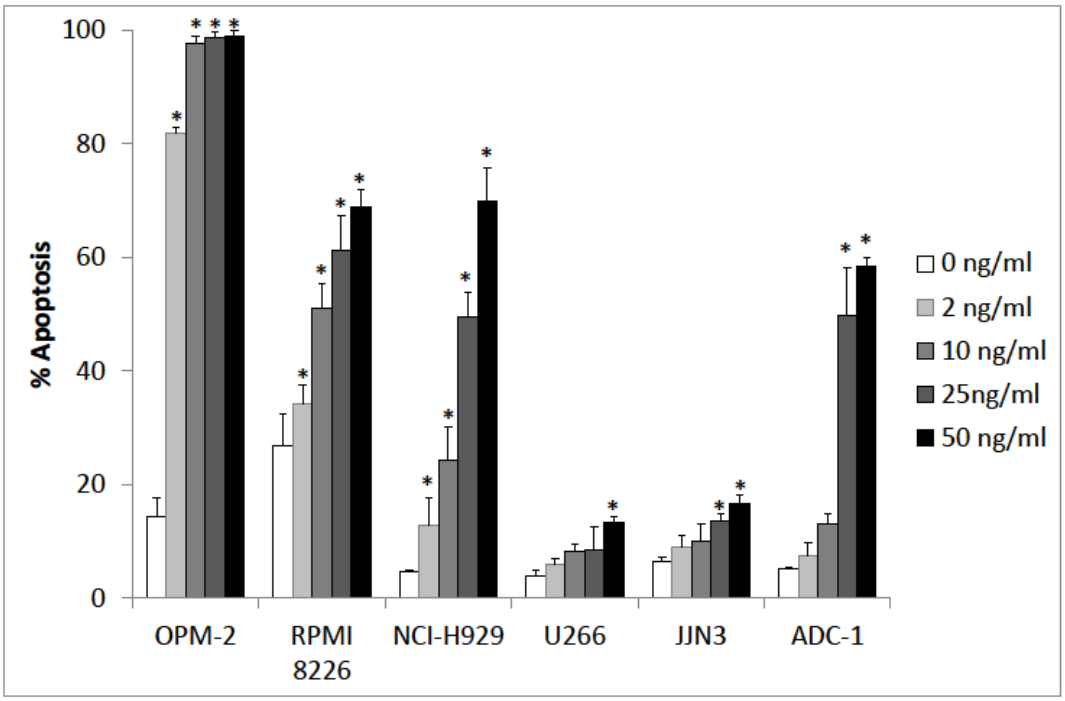


Figure 2*: The effect of apoptosis on the MM cell lines after the treatment with SAHA +/- TRAIL for 24h.* **(a)** *Morphological assessment of apoptosis using Hoechst 33342 /PI nuclear staining, after treatment shows SAHA synergistically enhances TRAIL apoptosis in U266 cell line. Apoptotic cells were identified by condensation and or fragmentation of the nucleus and intense staining while live cells were clear-edged, round, regular and uniformly stained nuclei. The white arrow indicates selected apoptotic cells. Red cells are permeabilised (necrotic) or late apoptotic if nucleus condensed.* ***(b)*** *The percentage of apoptotic MM cells treatment with SAHA for 24h was increased in a concentration-dependent manner ($p < 0.05$, ANOVA). Synergistic response was defined as comparison of combination treatment group with the sum of the effects of TRAIL alone + SAHA alone and significance determined by using by the ANOVA test (*=$p<0.05$).*

Fig 2a

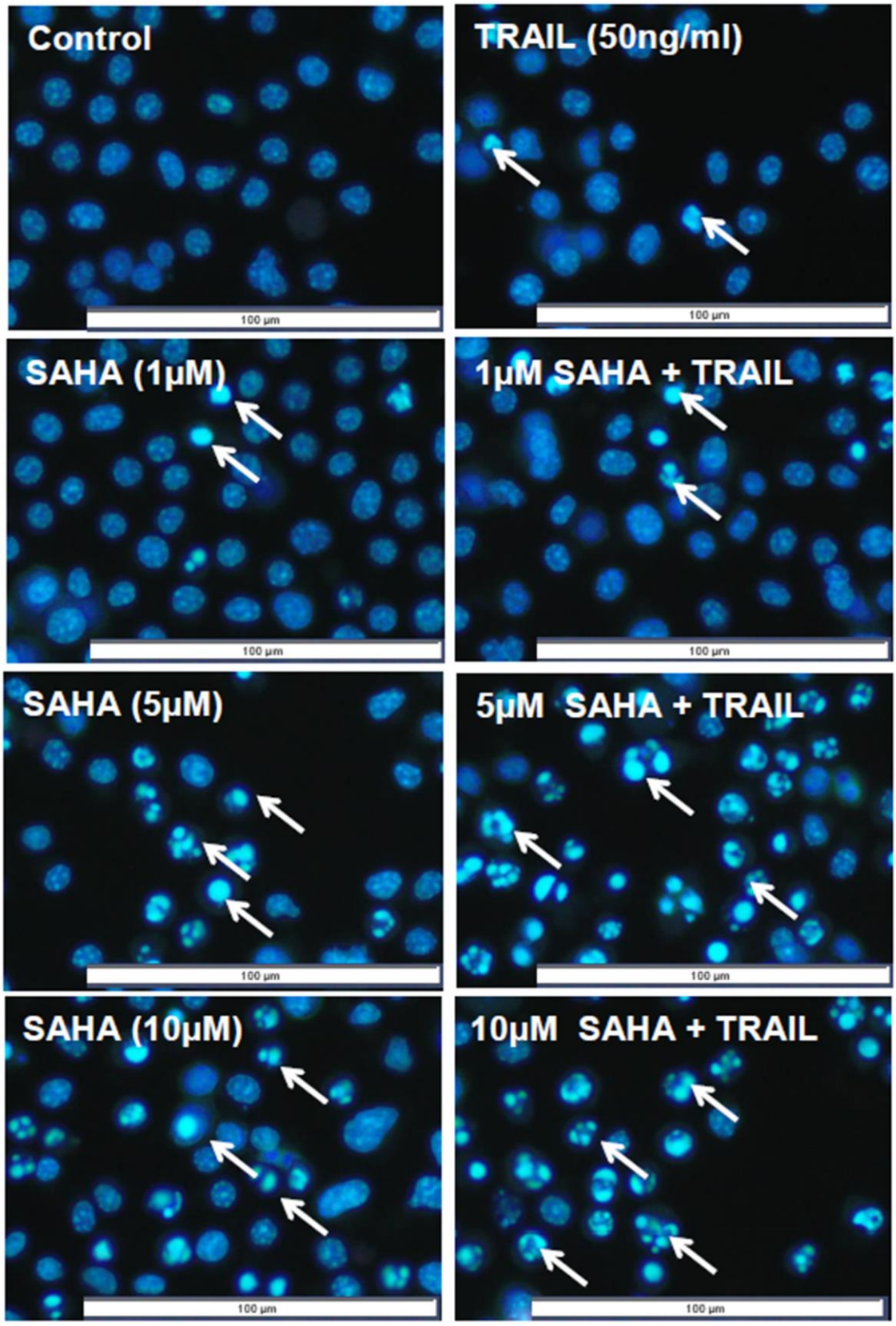
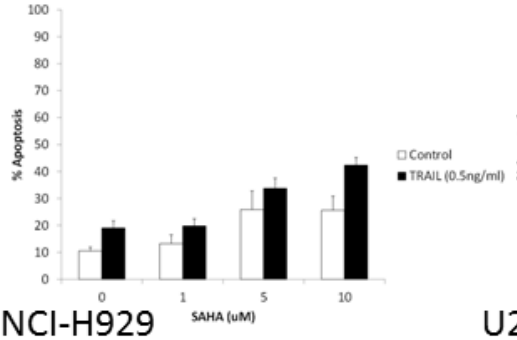
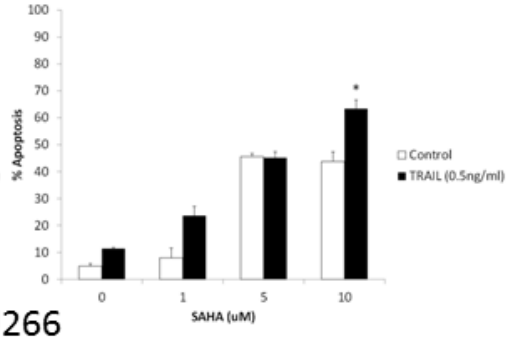

Control
TRAIL (50ng/ml)
SAHA (1μM)
1μM SAHA + TRAIL
SAHA (5μM)
5μM SAHA + TRAIL
SAHA (10μM)
10μM SAHA + TRAIL
100 μm

Figure 2B

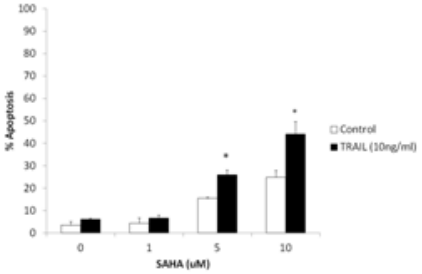
OPM2



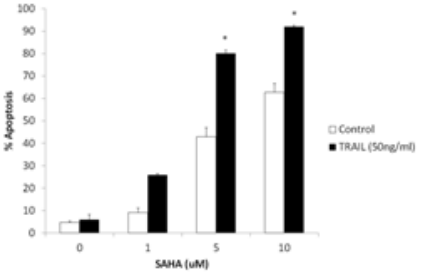
RPMI 8226



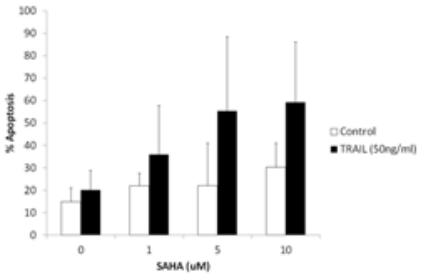
NCI-H929



U266



JJN-3



ADC-1

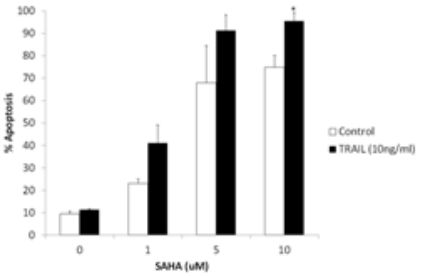


Figure 3: T*he effects of SAHA on myeloma cell viability, as evaluated by CellTiter-Glo® assay (i) OPM-2, (ii) RPMI 8226, (iii) NCI H929, (iv) U266, (v), JJN-3 and (vi) ADC- 1. The cells were treated as indicated for24 hours. Data was normalized to the vehicle control which was assigned 100% cell viability. The data is expressed as mean ± SD (three independent experiments, each in triplicate). Significance of combination treatment versus control is shown (ANOVA) where *=p<0.05.*

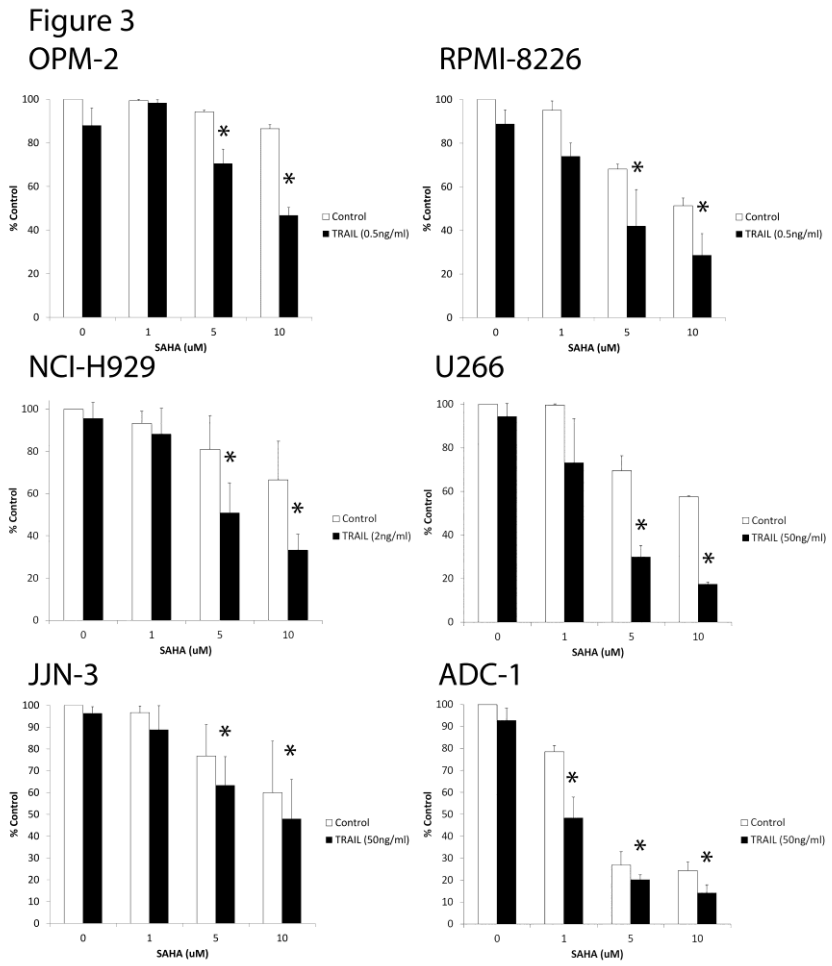
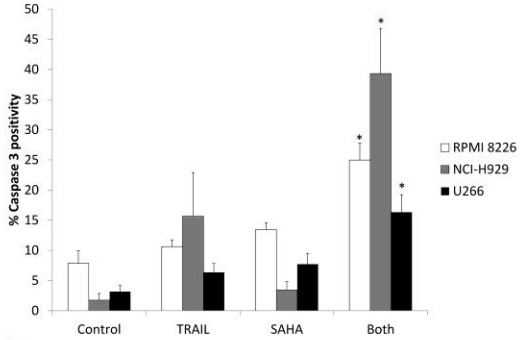


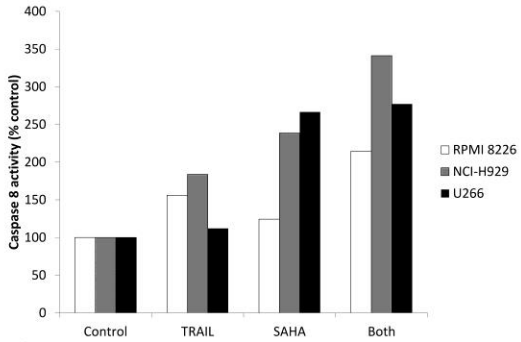
Figure *4a.* *Effect of SAHA+/- TRAIL on apoptosis of MM cell lines. Apoptosis was assessed using a Caspase-3 activity assay and analysed by flow cytometry. MM Cells were treated with the lowest doses of SAHA that appeared to enhance TRAIL-induced apoptosis as determined by Hoechst 33342 and PI staining (NCI-H929: 5μM SAHA, 10ng/ml TRAIL, RPMI 8226: 1μM SAHA, 2ng/ml TRAIL, U266: 5μM SAHA, 50ng/ml TRAIL). The data is expressed as average +/- standard deviation from three independent experiments, each in triplicate). Significance determined by using by the ANOVA test (*=p<0.05). Results confirmed the patterns of apoptosis induction seen by nuclear morphological assessment of apoptosis by Hoechst 33342 and PI staining in U266 and NCI-H929 and RPM 8226. B) Determination of caspase-8 and -9 activity in NCI-H929, RPMI 8226 and U266 cells treated with SAHA +/- TRAIL. TRAIL-sensitive cells show caspase-8 activation (NCI-H 929 (10ng/ml TRAIL and RPMI 8226 2ng/ml TRAIL) whereas U266 did not show TRAIL-induction at 50ng/ml. c RPMI activated caspase-9 after dual treatment whilst NCI-H929 potently activated Caspase-9 by SAHA alone. Results shown are mean of two independent analyses.*

Figure 4

a) Caspase-3



b) Caspase-8



c) Caspase-9

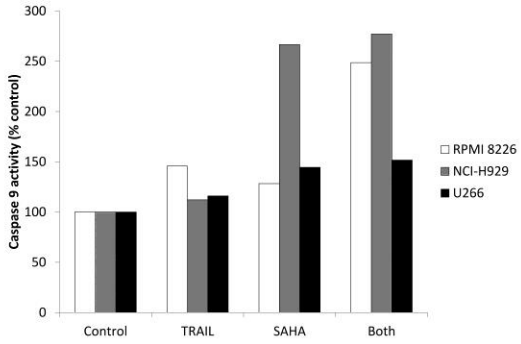


Figure 5*: Generation of* ***TRAIL*** ^{R} *population. a) Generation of* ***TRAIL-resistant NCI-H929 and RPMI 8226*** *cells by stimulating the TRAIL sensitive cells with a high/lethal dose of TRAIL (50 ng/ ml) and sub-culturing of surviving cells results in an increase in cell viability and significant reduction of the percentage of apoptosis in TRAIL resistant cells compared to parental TRAIL sensitive cells. b) SAHA does not synergistically enhance TRAIL responses in TRAIL-insensitive cell lines. Cells were treated with SAHA in the presence and absence of TRAIL resulting in significant reduction of the percentage of apoptosis in NCIH929 and RPMI 8226 TRAIL-resistant cells compared to parental TRAIL-sensitive cells (ANOVA *=p<0.05,).*

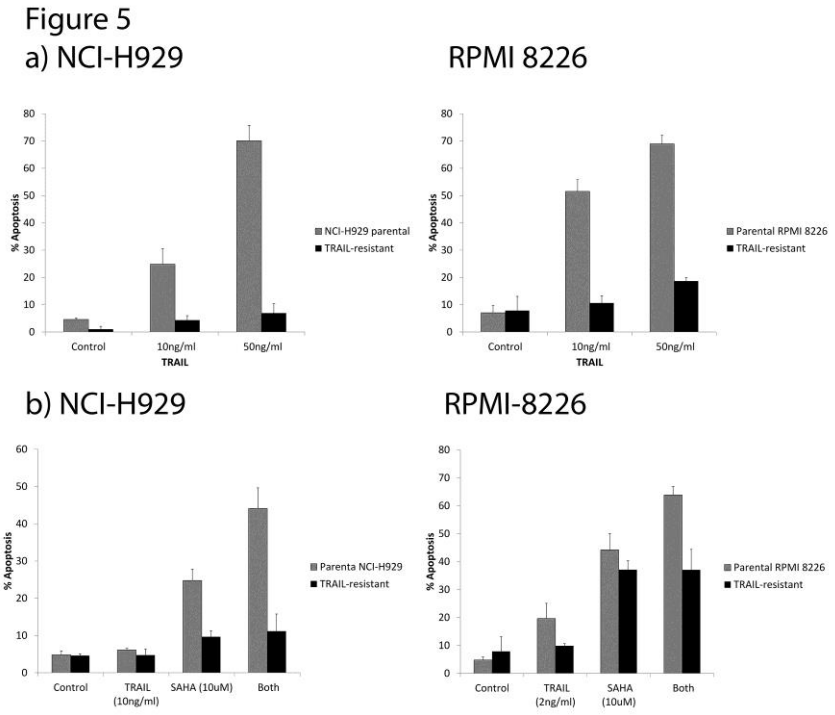


Figure 6: *Effect of SAHA +/- TRAIL on apoptosis of MM cell lines in suspension vs. 3D culture conditions. MM Cells were treated with SAHA +/- TRAIL determined by Hoechst 33342 and PI staining for24 h. The data is expressed as average and median (three independent experiments, each in triplicate). The statistical significance was determined by comparison with the vehicle control; $P < 0.05$ was considered statistically significant and determined by ANOVA test.* **b***: Induction of apoptosis in U266 cell lines following treatment with SAHA for 24hr in the 3D alginate model. Hoechst 33342 stain was used to demonstrate apoptosis cells fragmentation of the nucleus, intense staining, and chromatin condensation while live cells were clear-edged, round, regular and uniformly stained. White arrow indicates examples of apoptotic cells. Red Propidium iodide-stained cells with permeabilised membranes and condensed nuclei are late apoptotic.*

Figure 6

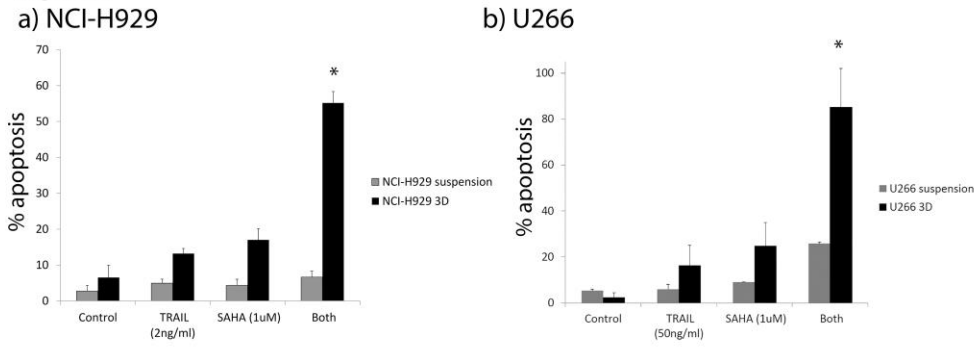
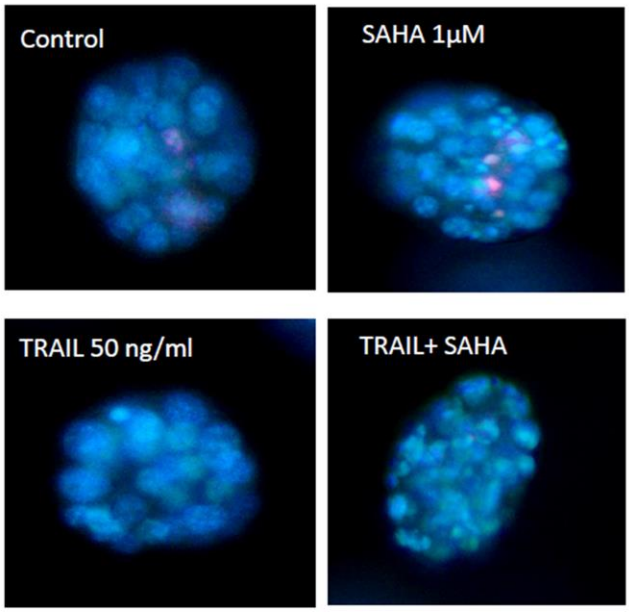
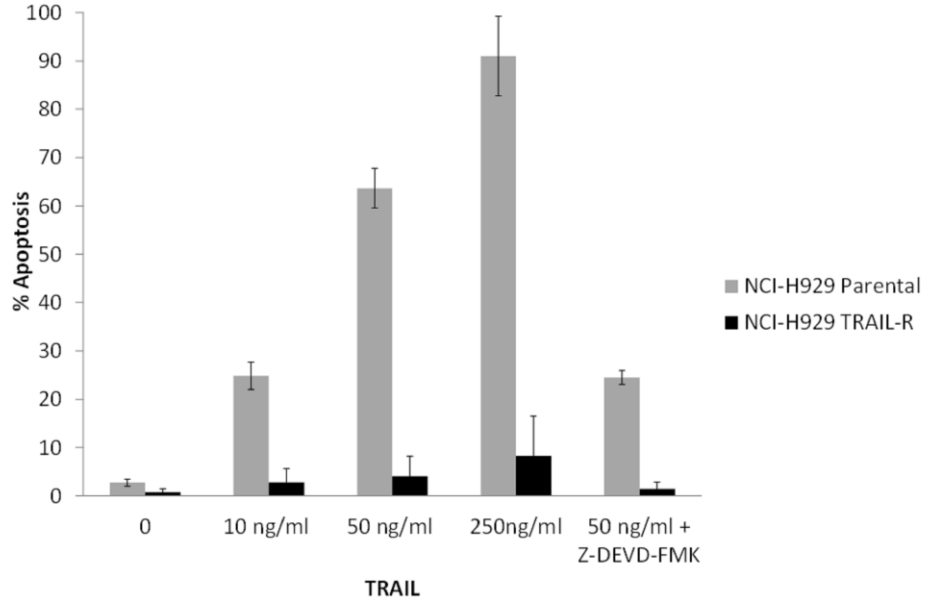


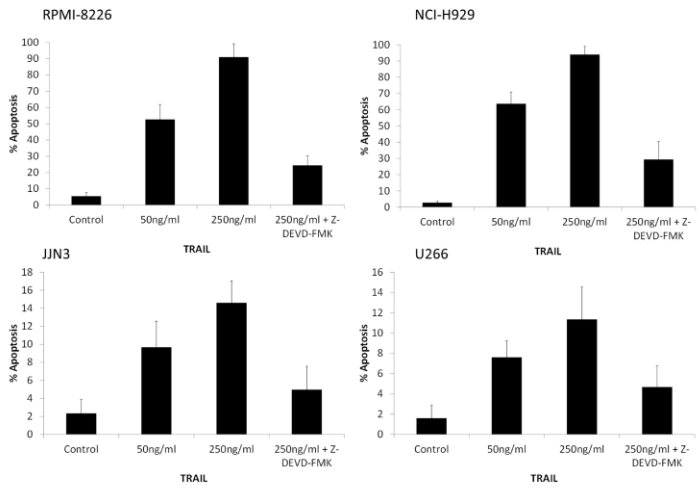
Figure 6b



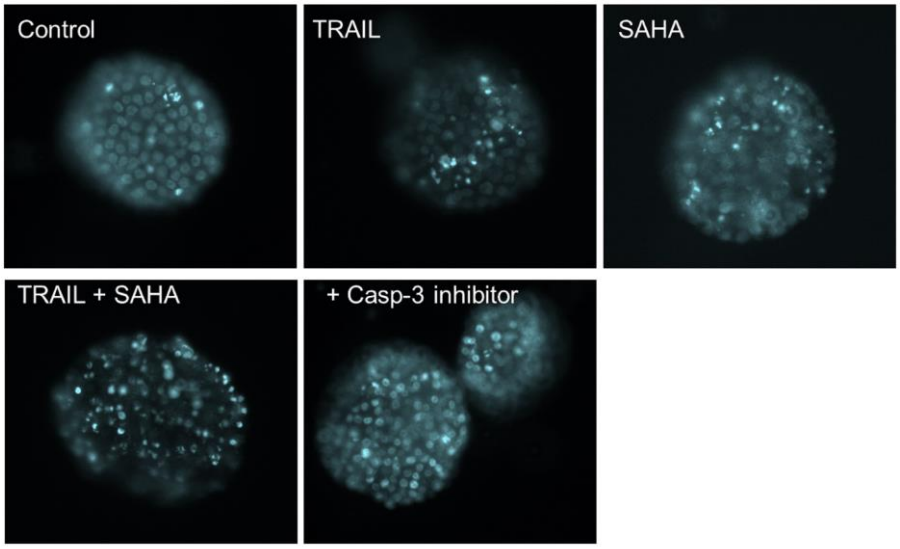
Supplementary figure 1. Cells were treated TRAIL (50ng/ml and 250ng/ml) and apoptosis determined by Hoechst 33342 staining and assessment of nuclear morphology. Cells treated with 250ng/ml TRAIL were co-incubated with 10μM of caspase-3 inhibitor Z-DEVD-FMK for 24 hours. All cells lines showed a dose-dependent increase in apoptosis up to 250ng/ml, and Z-DEVD-FMK partially reversed apoptosis levels to below those observed with 50ng/ml.



Supplementary figure 2. Parental and TRAIL-resistant NCI-H929 cells wer treated with TRAIL for 24 hours and apoptosis determined by Hoechst 33342 staining and assessment of nuclear morphology. TRAIL-resistant cells responded very weakly to 250ng/ml TRAIl (<10% apoptosis), however this was reversed by inclusion of 10μM the caspase-3 inhibitor Z-DEVD-FMK.



Supplementary figure 3. NCI-H929 cells were cultured in aginate beads fo 10 days followed by challenge with TRAIL, SAHA or combination treatment. 3D cell cultures were stained with Hoechst 33342 nuclear morphology assessed. Combination treatment induced apoptosis in the majority of cells, whereas cells co-incubated with the caspase-3 inhibitor Z-DEVD-FMK showed a complete inhibition of apoptosis.



Reference List

[1] A. Zaravinos, P. Kanellou, G.I Lambrou, D.A. Spandidos, Gene set enrichment analysis of the NF-κB/Snail/YY1/RKIP circuitry in multiple myeloma, Tumor Biol. 35 (2014) 4987-5005.

[2] J. Blade´ *, M. Teresa Cibeira, C. Fernandez de Larrea & L. Rosin˜ ol, Multiple myeloma, Annals of Oncology (Supplement 7): vii313–vii319,. 21 (2010).

[3] D. Lemancewicz, L. Bolkun, E. Jablonska, A. Kulczynska, U. Bolkun-Skornicka, J. Kloczko, J. Dzieciol, Evaluation of TNF superfamily molecules in multiple myeloma patients: correlation with biological and clinical features, Leuk. Res. 37 (2013) 1089-1093.

[4] W. Matsui, C.A. Huff, Q. Wang, M.T. Malehorn, J. Barber, Y. Tanhehco, B.D. Smith, C.I. Civin, R.J. Jones, Characterization of clonogenic multiple myeloma cells, Blood. 103 (2004) 2332-2336.

[5] G. Pan, K. O'Rourke, A.M. Chinnaiyan, R. Gentz, R. Ebner, J. Ni, V.M. Dixit, The receptor for the cytotoxic ligand TRAIL, Science. 276 (1997) 111-113.

[6] H. Yamada, S. Tada-Oikawa, A. Uchida, S. Kawanishi, TRAIL causes cleavage of bid by caspase-8 and loss of mitochondrial membrane potential resulting in apoptosis in BJAB cells, Biochem. Biophys. Res. Commun. 265 (1999) 130-133.

[7] T. Doi, H. Murakami, A. Ohtsu, N. Fuse, T. Yoshino, N. Yamamoto, N. Boku, Y. Onozawa, C. Hsu, K. Gorski, Phase 1 study of conatumumab, a pro-apoptotic death receptor 5 agonist antibody, in Japanese patients with advanced solid tumors, Cancer Chemother. Pharmacol. 68 (2011) 733-741.

[8] R.S. Herbst, S.G. Eckhardt, R. Kurzrock, S. Ebbinghaus, P.J. O'Dwyer, M.S. Gordon, W. Novotny, M.A. Goldwasser, T.M. Tohnya, B.L. Lum, A. Ashkenazi, A.M. Jubb, D.S. Mendelson, Phase I dose-escalation study of recombinant human Apo2L/TRAIL, a dual proapoptotic receptor agonist, in patients with advanced cancer, J. Clin. Oncol. 28 (2010) 2839-2846.

[9] J. von Pawel, J.H. Harvey, D.R. Spigel, M. Dediu, M. Reck, C.L. Cebotaru, R.C. Humphreys, M.J. Gribbin, N.L. Fox, D.R. Camidge, Phase II Trial of Mapatumumab, a Fully Human Agonist Monoclonal Antibody to Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand Receptor 1 (TRAIL-R1), in Combination With Paclitaxel and Carboplatin in Patients With Advanced Non–Small-Cell Lung Cancer, Clinical lung cancer. 15 (2014) 188-196. e2.

[10] C. Geng, J. Hou, Y. Zhao, X. Ke, Z. Wang, L. Qiu, H. Xi, F. Wang, N. Wei, Y. Liu, A multicenter, open- label phase II study of recombinant CPT (Circularly Permuted TRAIL) plus thalidomide in patients with relapsed and refractory multiple myeloma, Am. J. Hematol. 89 (2014) 1037-1042.

[11] Y. Jian, Y. Chen, C. Geng, N. Liu, G. Yang, J. Liu, X. Li, H. Deng, W. Chen, Target and resistance-related proteins of recombinant mutant human tumor necrosis factor-related apoptosis-inducing ligand on myeloma cell lines, Biomedical Reports. 4 (2016) 723-727.

[12] J. Abdulghani, J.E. Allen, D.T. Dicker, Y.Y. Liu, D. Goldenberg, C.D. Smith, R. Humphreys, W.S. El-Deiry, Sorafenib sensitizes solid tumors to Apo2L/TRAIL and Apo2L/TRAIL receptor agonist antibodies by the Jak2-Stat3-Mcl1 axis, PloS one. 8 (2013) e75414.

[13] S. Deleu, E. Menu, E. Van Valckenborgh, B. Van Camp, J. Fraczek, I.V. Broek, V. Rogiers, K. Vanderkerken, Histone deacetylase inhibitors in multiple myeloma, Hematology Reviews. 1 (2009).

[14] C.L. Peterson, M. Laniel, Histones and histone modifications, Current Biology. 14 (2004) R546-R551.

[15] T. Hideshima, K.C. Anderson, Histone deacetylase inhibitors in the treatment for multiple myeloma, Int. J. Hematol. 97 (2013) 324-332.

[16] A. Younes, J.G. Berdeja, M.R. Patel, I. Flinn, J.F. Gerecitano, S.S. Neelapu, K.R. Kelly, A.R. Copeland, A. Akins, M.S. Clancy, Safety, tolerability, and preliminary activity of CUDC-907, a first-in-class, oral, dual inhibitor of HDAC and PI3K, in patients with relapsed or refractory lymphoma or multiple myeloma: an open-label, dose-escalation, phase 1 trial, The Lancet Oncology. 17 (2016) 622-631.

[17] R. Niesvizky, S. Ely, T. Mark, S. Aggarwal, J.L. Gabrilove, J.J. Wright, S. Chen- Kiang, J.A. Sparano, Phase 2 trial of the histone deacetylase inhibitor romidepsin for the treatment of refractory multiple myeloma, Cancer. 117 (2011) 336-342.

[18] Sayers, Thomas and Cross, Neil, Triggering death receptors to promote
cancer cell death, in: Anonymous , 2014.

[19] N. Al-Yacoub, L.F. Fecker, M. Möbs, M. Plötz, F.K. Braun, W. Sterry, J. Eberle, Apoptosis induction by SAHA in cutaneous T-cell lymphoma cells is related to downregulation of c-FLIP and enhanced TRAIL signaling, J. Invest. Dermatol. 132 (2012) 2263-2274.

[20] G. Matthews, M. Lefebure, M. Doyle, J. Shortt, J. Ellul, M. Chesi, K. Banks, E. Vidacs, D. Faulkner, P. Atadja, Preclinical screening of histone deacetylase inhibitors combined with ABT-737, rhTRAIL/MD5-1 or 5-azacytidine using syngeneic Vk* MYC multiple myeloma, Cell death & disease. 4 (2013) e798.

[21] T.E. Fandy, S. Shankar, D.D. Ross, E. Sausville, R.K. Srivastava, Interactive effects of HDAC inhibitors and TRAIL on apoptosis are associated with changes in mitochondrial functions and expressions of cell cycle regulatory genes in multiple myeloma, Neoplasia. 7 (2005) 646-657.

[22] D.F. Jelinek, Myeloma research goes 3D, Blood. 112 (2008) 2600-2601.

[23] C.L. Le Maitre, A.J. Freemont, J.A. Hoyland, The role of interleukin-1 in the pathogenesis of human intervertebral disc degeneration, Arthritis research & therapy. 7 (2005) 1.

[24] S. Vitovski, A.D. Chantry, M.A. Lawson, P.I. Croucher, Targeting tumour-initiating cells with TRAIL based combination therapy ensures complete and lasting eradication of multiple myeloma tumours in vivo, PloS one. 7 (2012) e35830.

[25] K. Kagawa, A. Nakano, H. Miki, A. Oda, H. Amou, K. Takeuchi, S. Nakamura, T. Harada, S. Fujii, K. Yata, Inhibition of TACE activity enhances the susceptibility of myeloma cells to TRAIL, PloS one. 7 (2012) e31594.

[26] R.R. Rosato, J.A. Almenara, Y. Dai, S. Grant, Simultaneous activation of the intrinsic and extrinsic pathways by histone deacetylase (HDAC) inhibitors and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) synergistically induces mitochondrial damage and apoptosis in human leukemia cells, Mol. Cancer. Ther. 2 (2003) 1273-1284.

[27] McStay, GP, Salvesen, GS and Green, DR. Overlapping cleavage motif selectivity of caspases: Implications for analysis of apoptotic pathways. Cell Death and Differentiation 15 (2008) 322-331.

[28] C. Fischbach, R. Chen, T. Matsumoto, T. Schmelzle, J.S. Brugge, P.J. Polverini, D.J. Mooney, Engineering tumors with 3D scaffolds, Nature methods. 4 (2007) 855-860.

[29] M. Amatangelo, A. Garipov, H. Li, J.R. Conejo-Garcia, D. Speicher, R. Zhang, Three-dimensional culture sensitizes epithelial ovarian cancer cells to EZH2 methyltransferase inhibition, Cell Cycle. 12 (2013) 2113-2119.

[30] X. Xu, C. Liu, Y. Liu, L. Yang, N. Li, X. Guo, G. Sun, X. Ma, Enrichment of cancer stem cell-like cells by culture in alginate gel beads, J. Biotechnol. 177 (2014) 1-12.